THE TANDEM SCANNING REFLECTED LIGHT MICROSCOPE

M. Petran[1], M. Hadravsky[1], A. Boyde[2]

[1]Dept. of Biophysics, Faculty of Medicine, Charles University of Prague at Pilsen
Karlovska 48, PLZEN 30167, Czech Republic
[2]Dept. of Anatomy and Embryology, University College London, Gower St., London WC1E 6BT, England

A b s t r a c t. Reflected light microscopy of biological material has been a very difficult task and many different but hardly successful attempts have been made to get usable images. The main reasons for this state are: weak reflections from the biologically important structures in the object as against strong reflection at its surface; reflections at optical surfaces in the microscope which cause a deterioration of contrast; and the mixing together of reflections from many levels in the object so that the signal coming from the focussed-on level is lost in noise and d.c. components.

We tried, and successfully, to reverse this situation and to make it possible for the signal to overwhelm spurious and scattered light using double, or, as we call it, tandem scanning. The object is illuminated only in small patches lying in one plane and moving across this plane and only the light reflected from these illuminated patches is allowed to pass into the image plane and participate in image formation. The image consists of points which travel over the image plane and which are geometrical images of the illuminated patches in the focussed-on object plane. To ensure that only the light belonging to these geometrical images be allowed to enter the image, the image plane is covered with an opaque diaphragm having holes in locations exactly corresponding to the locations of the geometrical images of the illuminated object points; this diaphragm travels in concordance with the first scanning and so the complete image of the focussed-on object plane is formed successively.

Both scans are performed by a single device, a Nipkow disc carrying in its annular periphery several ten thousands of holes arranged in Archimedean spirals. The disc is 100 mm in diameter and rotates about 100 rpm driven by an electric motor. On one side the disc is illumi-nated in a circle 18 mm in diameter, and the light trans-mitted through several hundred holes and reflected in a mirror system passes a microscope objective which forms the images of the disc holes in the object plane. The light reflected here passes through the same objective and mir-ror system (one mirror being a 'limitlessly thin' beam splitter) to pass conjugate aperture holes on the observa-tion side of the Nipkow disc. As the disc lies at the inter-mediate image plane of the objective lens on both its illuminating and observation sides, only light emanating from reflection or fluorescence in the plane of focus can contribute to the image. High contrast images of very thin focussed-on layers are thus formed.

The practical arrangements are such that very large specimens can be examined: the specimens for this micro-scope need not themselves be microscopic.

K e y w o r d s: scanning reflected light microscope

THE PRESENT STATE OF MICROSCOPY IN LIVING TISSUES

May of our readers may already have made attempts to observe some tissue in a living animal, yet been completely disappointed. In most cases nothing other than a general pink hue can be seen, in some places decorated with brilliant reflections from the wet object surface. Better results may, however, be obtained when using traditional reflected light microscopes with plant tissues.

This disappointing experience has led to the use of special, unique preparations for any

particular cell or tissue type, e.g., living nervous cells are observed in retina dissected from stripped chicken eyes, blood vessels and nerves are observed in mammalian mesentery, etc. Other special preparation techniques and microscopic devices have also been developed, such as injections, squashes, etc,. the slit lamp in ophthalmology, the Ultropak microscope objectives by Leitz etc., which all may help in some cases, but fail in most.

The phase contrast microscope has been an important achievement, but its use in biology is limited to microscopy of individual cells and thin sheets of tissue accessible from both sides (phase contrast reflected light microscopy of planar, e.g., metal surfaces is possible). For practical purposes, electron microscopic methods can only work with dry specimens of animal and plant tissues.

Acoustic and X-ray microscopy are in their infancy and it would be daring to predict their future in the biological sciences.

The reasons for the unsatisfactory state of affairs for the light microscopical observations of bulk biological objects and bulk objects in general are evident. We have to illuminate from above, so that in the case of high power objectives the only practicable way is to illuminate through the same objectives which is used for observation. Too little of the illuminating light reaches the object layer which is to be visualised, and too much is reflected back into the image plane by lens surfaces and by the object surface, thus degrading the image contrast.

All these reflections can be prevented. The reflections inside the microscope can be suppressed by polarization. If the illuminating light is linearly polarised in one direction, a perpendicularly crossed analyser in the eyepiece will not transmit light which is reflected inside the microscope. If a quarter wave plate is placed in front of the objective, light which passes through it to illuminate the specimen can pass back quite freely, so that reaching the analyser it passes freely into the eyepiece. The reflections from the object surface can be suppressed using an immersion objective. These

means have been provided in recent decades by many first class manufacturers, but in biological specimens the improvement achieved does not pay for the effort. Other manufactures have tried to eliminate these effects by illuminating through one part of the objective aperture and observing through another. Still others do not illuminate through the objective proper, but use a special illuminating channel surrounding the observation objective (e.g., Leitz's Ultropak). For animal tissues, the effect is also scanty. The general insufficiency of all these approaches has common and deep reasons lying in the following points.

The illuminating light penetrates deeply into the semitransparent substance of the object, so that light is reflected by structures in a very thick layer of which only a very thin sheet is actually focussed on. Thus the image of this thin layer is blurred by unsharp images of the layers above and below. This effect is often exaggerated because we usually illuminate an object field substantially broader than the usable image size.

The importance of the points just mentioned can easily be demonstrated experimentally if we limit the illuminated field. On narrowing the field diaphragm we see a smaller and smaller area but with ever improving contrast. The area fully illuminated is surrounded with a halo with luminosity decaying towards the periphery.

Let us discuss the possibilities of exploiting this phenomenon in practice. Immediate use cannot take place, since on limiting the field, we lose important information concerning the connections of the observed field to its surroundings. This information can be obtained, at least theoretically, by also successively observing these surroundings with equally limited illumination. On doing so, however, we must also consider the halo surrounding the illuminated and observed spot. This halo contains light reflected from layers of the specimen lying above and below the layer which contains the observed central spot. Hence it follows that a simple scanning by motion of the diaphragm limiting the field cannot be used. The halos, being much

broader than the central spots, would sum and degrade the cumulative image to an extent which would make it of the same quality as an untreated image of a broader field.

Nevertheless, there are at least three possible approaches making use of the phenomenon just described: (a) move the diaphragm manually and draw a compiled image by hand; (b) photograph the individual images, and using scissors to isolate only the central spots, mount a set of such images together to form an image of a broader area; (c) isolate the central spot of an elementary image using a diaphragm in the image field corresponding in size and position to the object area illuminated, and scan this in concordant motion with the illuminating diaphragm. This last approach is the one used in tandem scanning microscopy [12].

Means for tandem scanning

Let us consider different possibilities for the technical realization of the ideas outlined above, and their advantages and disadvantages.

Moving the object - static beam

The most straightforward way is the following [25]: the object is illuminated with a single and the smallest possible spot (having dimensions near to the Airy disc) lying on the optical axis of the objective. Light reflected by the object is (via a semireflecting mirror) filtered through a limitlessly small opening conjugate to the illuminated point. Light passing this opening is accepted by a photomultiplier, and converted into an electric signal. Scanning is accomplished by moving the object stage mechanically and the image is displayed on a cathode ray tube, the ray deflections of which are derived from the scanning motions of the stage. The CRT display brightness is modulated by the signal detected by the photomultiplier. The light source is usually a laser. Demands on the microscope objective are very moderate: only spherical aberration must be corrected unconditionally. Correction for chromatic aberration is of advantage only when the same field should be visualised subsequently in different wavelengths. Very high demands must

be satisfied in the stage driving mechanism. The necessary accuracy can only be attained when scanning is slow - several seconds (or more) for one complete image. If the scanning motion is not linear, a computer must be used to linearize the image; usually it is also used to memorize the image and display it continuously. A big advantage of this approach is the possibility of reaching an absolute limit in resolution performance - of course only in absolutely rigid and unchanging objects. The device would not be useful for soft, flexible living biological object.

Moving both fields - scanning the optics mechanically

The difficulties encountered in scanning with movements of the stage can be circumvented by scanning with a single moving light beam pencil (NBS Spec. Pub. 400-24, 1977; [24]). Although it is not less difficult technologically, it poses no restrictions on the features of the object. The objective have to be corrected for a broad field. The image forming time can be a little shorter than in the previous case. The electronic supporting and auxiliary devices needed are approximately the same as above. Direct photography is again not possible because of the changing velocity of the scan and harmonic action. Whether scanning by moving the object (a) or the optical beam (b) one has to encounter problems of optoelectronic and electro-optic signal conversions. There are problems with the illuminating power, and a laser source is used, so that one has both the disadvantages as well as the advantages of monochromatic light.

Electronic scanning

Another possibility is to get rid of any complicated mechanical and electromechanical devices and to scan purely electronically. Illumination scanning can be accomplished with a cathode ray tube as in flying spot microscopy [27]. Illumination scanning can also be achieved in an SEM, with the advantage of using all the existing electronics and signal detection facilities which are already a part of such a complex. As far as

we know, however, this 'flying spot' concept has not yet been applied to achieve tandem or 'confocal' scanning, perhaps because it is rather difficult to address simultaneously the optically conjugate points of two cathode ray tubes, that used for illumination and the iconoscope.

Mechanical tandem scanning

Still another possibility is to get rid of any electronics and to scan mechanically and linearly both conjugate fields and in this way display the image purely optically. The simplest linear device for mechanical scanning is the Nipkow disc patented 100 years ago [11]. It is a rotating opaque disc carrying holes arranged on an Archimedean spiral. In this case it is impractical to illuminate with a laser and illumination with a conventional light source is too weak when a full theoretical advantage of extremely small holes is to be taken. To make this approach usable, many holes can be used to scan the illumination and image fields simultaneously. The contrast in the device is compromised to a small extent in this way, but another great advantage can be gained: the fields are subdivided into many strips each of which is scanned several times per revolution of the disc so that several complete images are formed during one revolution. Thus the image forming time can be reduced to less than one-twentieth of a second, permitting the formation of real time images and allowing the observation of moving (biological) objects. Illumination and image scannings may be accomplished either with two synchronized Nipkow discs, or with a single disc, using one side of it for the illumination and the opposite side for image formation. The disposition of optical elements must fulfil some strict geometrical and kinematic conditions which we shall discuss later.

In devices of this kind, it is important that the highest demands on accuracy of correspondance of both scans are fulfilled solely with mechanical means, which are much more stable in time than electronics. However, because no transformations of optical signals into electrical ones and vice versa take place, no additional noise sources are present.

General remarks about tandem (confocal) scanning microscopes

The story of all these microscopes has been broadly discussed by Wilson and Sheppard [26], who call them Type II confocal microscopes; other possible names would be twice scanning microscopes, or double scanning microscopes or scanning microscopes with image filtering or scanning microscopes with delta-filtered image. Our 'Tandem Scanning Reflected Light Microscope' (TSRLM) is one member of this family and the name has taxonomic priority. The theory of all these microscopes is explained by Wilson and Sheppard [26]. We will describe the practical and technological aspects.

All such microscopes may be realized by two physically substantially identical systems, the first of which forms an image of one or more pointlike light sources in the specimen, whilst the second one forms an image of these points, the luminosity of which had been influenced by the transparency of the object. Such devices could be called transmitted light Type II confocal microscopes [22]. For such transmitted light microscopes the simplest construction would be scanning with a single spot performed with mechanical movements of a thin object slice lying in between two coaxial objectives (as in above *Moving the object - static beam*). Only such an arrangement can use two objectives which are not exactly identical, and only in this way a substantial enhancement of resolution can be achieved relatively simply, and paying little attention to an exact matching of objective powers and aberrations (especially the field curvatures do not need to be corrected). The arrangement allows a greater freedom for differences in refractive indices and thicknesses of object carrier and cover glasses, embedding media and immersion liquids.

Any of the other designs outlined above need selected flatfield objectives and exact matching of indices and thicknesses of all the media between the object and the bottom and top objectives - tasks rather difficult to fulfil.

The other possibility [12] is to utilise the principle of autocollimation, and to use a single device for illumination and image formation. Such an instrument would be called reflected light Type II confocal microscope and can employ any of the above designs. All have been realized and all give good results. Except for our Tandem Scanning Reflected Light Microscope, all devices must convert their optical signal into an electrical one, which, after processing, has to be converted back into an optical image. There are good reasons for making such a detour: (i) the reflected light (optical signal) is usually extremely weak so that it is practical to use a more sensitive detector than the unaided eye: (ii) the complete image scanning and reconstruction last several seconds, much longer than is permissible for a steady image impression, so that an image display tube becomes a necessity: (iii) most kinds of mechanical and opto-mechanical scans are non-linear, which could lead to geometrical and/or image luminance distortions if not corrected prior to display: and, (iv) it is often desirable to display mathematically processed image information.

Such a detour is made unnecessary in our TSRLM, which leads to a much simpler device, both in principle and in practice. Scanning with a Nipkow disc is linear, and if scanned with more holes (in our case several hundreds in the field of view), the image luminosity is proportionally enlarged and the image repetition frequency can be high enough (20 full images per second composed of 400 interlaced images per second) to give a quiet steady image like cinema or television. If digital image processing is necessary, it can be conveniently made with an attachment placed behind the microscope proper.

CONSTRUCTION OF THE TSRLM

The scanning device - a modified Nipkow disc [11] is a thin metallic foil held stretched by a 100 mm-diameter hoop. The foil is perforated by 48,000 holes in an annular region 18mm broad. The holes are arranged on Archimedean spirals and form a quasi-hexago-

nal pattern (Fig. 1). Diametrically opposite holes are on exactly identical radii. The pattern as a whole possesses a central symmetry.
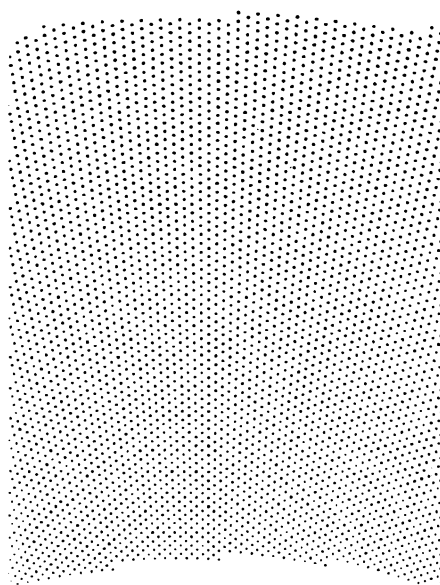

Fig. 1. Pattern of holes.

The disc is mounted perpendicularly to and exactly centred on an axis of revolution.On the same axis is the rotor of a collector-less electric motor. The disc is evenly illuminated in a circular area 18 mm in diameter on one side, with rays converging on the entrance pupil of the microscope objective. A system of four mirrors, one of them semi-reflecting and extremely thin, is situated between the disc and the objective (Figs 2 and 3, explanation below).
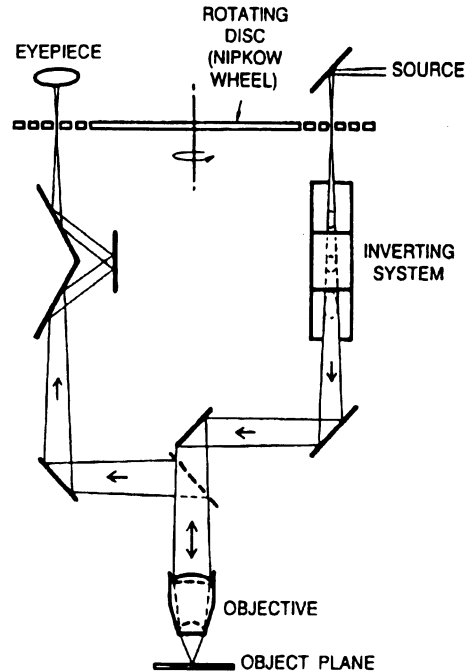
The microscope objective forms images of the illuminated disc holes into the object plane to be observed. Light reflected by the object enters the same microscope objective and forms images of all the object structures illuminated. These images lie at different distances behind the objective depending on the position of object pints. However, one set of object points has been illuminated most strongly, namely those points in which the images of the Nipkow disc holes have been formed by the objective. Images of these strong light spots lie in the plane of the Nipkow disc on the side opposite to the illuminated area.
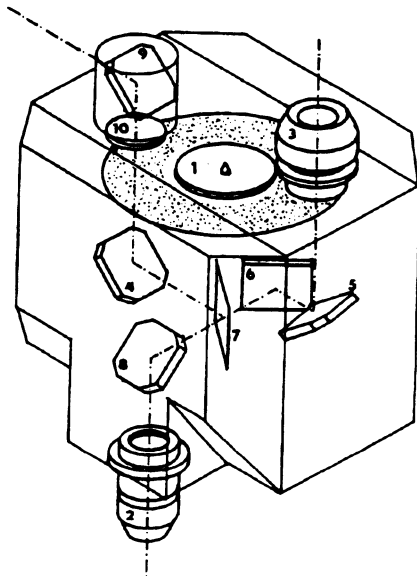
Fig. 2. Diagram showing the essential optical components in the 'Confocal 2002' TSM from Pilsen (Plzen). 1. Rotating Nipkov disc. 2. Objective. 3. Eyepiece. 4.5.6.7. Internal mirrors. 7. Thin semi-transparent mirror. 8. Output mirror. 9. Input mirror. 10. Field lens.

Fig. 3. A schematic diagram of the tandem scanning microscope.

The function of the mirror system is to localize these images exactly into the holes of that area. If the mirror adjustment is perfect, only the light originating in Nipkow disc holes in the field of illumination and reflected in the focussed-on plane of the object passes the holes in the image area of the Nipkow disc quantitatively. Almost all light reflected elsewhere - whether within the specimen or on the optical components - is stopped on the opaque portions between the holes.

The 'macroscopic' function of the mirror system may be explained as follows. The two systems of holes filtering the illumination and the image must be identical, but the Nipkow disc pattern is centrally symmetrical. The mirrors have to transform this central symmetry virtually into congruency. If we look through the microscope objective in the direction towards the Nipkow disc, we have to see that the pattern of the illumination exactly coincides with the pattern of the image area, which coincidence must be preserved even during the rotation of the disc. The exact, continuous and permanent virtual overlapping of images of

any two functionally coupled holes is a task which has to be met precise fabrication of the disc, and by centering and adjustment of the disc's engine and bearings, and positioning of the four mirrors. The semi-reflecting mirror must be extremely thin in order not to double the images and not to introduce astigmatism. Light traps must be provided to limit light scattering on internal walls.

The heart of the TSRLM has been described in the preceding paragraphs. However, there are some further considerations regarding its orientation in space.

The Nipkow disc axle should be vertical for the sake of mechanical stability. The objective axis should be vertical because immersion objectives are preferred. The light input has to be oriented horizontally to limit the vertical dimensions of the illuminating device. If arranged in such a way, there must be one more mirror before the Nipkow disc in the illuminating branch and one mirror preceding the objective (Fig. 3).

The illuminating device can be engineered in several ways. A uniformly radiating, broad area source (such as a tungsten ribbon lamp, radiofrequency heated carbide disc, or simply the sun) can be used, the image of which is formed exactly in the plane of the Nipkow disc (almost incoherent illumination).

Alternatively, a very bright arc discharge lamp (highest pressure mercury, xenon, or metal halides) may be used for coherent illumination, in which case the image of the arc is to be formed in the objective entrance pupil, that is near to its 'internal' focal plane. An image of the aperture of a condenser lens is formed in the plane of the Nipkow disc by a relay lens. In both cases care must be taken that all ray pencils emerging from the Nipkow disc holes hit the objective entrance pupil.

The optical components of the microscope proper may be conventional, but must be sensibly chosen. The eyepieces must be Ramsden-type, i.e., the image plane must be in front of the lens because the Nipkow disc must lie in it.

Because the signal-carrying light emerges from the specimen in the same direction as the light reflected and scattered on lenses and surfaces in the specimen, immersion objectives are to be used throughout, and are to be well anti-reflection coated. For other considerations concerning objectives, see below.

IMAGE FEATURES

Theory predicts [22] and experiment confirms [8] improvement of resolution and of other image quality criteria in Type II confocal scanning microscopes. These conclusions are to a great extent valid also for the TSRLM. But to be exact, the theory must deal with fictional objects of exactly defined properties which are very rarely encountered in practice. Although the knowledge of this theory is important for the development and improvement of such microscopes, the user is interested in other, simpler questions. Which features characterize the images in TSRLM? In which respects do they differ from the images in a conventional microscope? For which samples is the TSRLM preferable to other types of microscope? Can contrast enhancing techniques be used in TSRLM? To what depth can the TSRLM penetrate? etc. We now try to give answers based ont on theory (although they can be deduced from it), but on our experience with existing instruments and different classes of specimen.

Type of specimen

1. Thin histological sections and thin, low absorption sheets.

For such objects, the TSRLM has hardly any advantages compared with methods such as transmitted light phase contrast or interference contrast, The predicted improvement in resolution has not yet been observed due to imperfections in the Nipkow disc pattern, To get contrast and resolution comparable to the above-mentioned techniques, the translucent objects must lie on thick homogeneous transparent supports, the refractive index of which is matched to that of the object.

If objects with these properties are not accessible for transmitted light microscopy, they may be considered as a special case in the following group.

2. Thick highly translucent, or transparent, three-dimensional objects with a homogeneous matrix in which details differing in refractive index or in absorption are dispersed. Examples: living animal and plant tissues, living or fossil bones and teeth, rocks containing microfossils.

For most of such objects the TSRLM has been the first device which has permitted their microscopical examination. In such objects light is reflected at discontinuities of refractive index (its real and imaginary components) as expressed by Fresnel formulae. But these discontinuities are ubiquitous and immense in number. For example, in biological tissues they surround every cell and each of its organelles and the strongest of them is the surface of the specimen, which is rarely of interest. The surface reflection is suppressed by the immersion liquid. A selection of the other reflecting details must be made from a comprehensible subspace of the whole object, usually a thin

layer comparable to a conventional histological thin section. Because, in the TSRLM, only a thin layer is fully illuminated and only the light reflected in this same layer is permitted to enter the image, it is ideally adapted for this purpose. It produces images of thin sections without the necessity to section and allows the study of serial 'sections' without the necessity to change slides or even to move the stage. These serial sections are matched to each other automatically, since it is enough to take pictures and move by small steps in the Z direction.

The depth limitation of the focussed-on layer is amazing, e.g., for a 40X 0.75NA water immersion objective we usually get a completely changed image every 5 μm. With a 1.3 NA lens, the image changes with a less than 1 μm focus change. To appreciate fully the significance of these possibilities, one must consider not only the acceleration of work, but the fact that it is not possible, or not permissible, to obtain serial sections with many classes of object, including museum specimens in general.

Some general features of the 'optical sectioning' must be mentioned. The effective thickness of a visualized layer depends strongly on the objective aperture: the higher the aperture, the thinner the section. The depth to which the microscope can penetrate depends on many features of the objective and of the object. It is limited physically by the free working distance of the objective, which depends strongly on the objective aperture and power. The image quality deteriorates slowly as we go deeper and deeper, due to the deformation of light wave fronts in the layers through which the light must propagate and which are not ideally homogeneous. The rate of this deterioration again depends on the objective aperture and power, but also on the optical quality of the specimen.

As a rule of thumb it may be stated that with a 40X 0.75 water immersion objective we may observe undeteriorated images to a depth of 0.2 mm in most wet animal tissues, and to see something up to its free working distance

of about 0.5 mm.

The TSRLM image is unfamiliar and different from those produced by conventional optical microscopes. In histological sections staining and microchemical reagents are generally used to reveal the chemical nature of the object's constituents. In living objects phase contrast microscopy and differential interference devices show optical path differences in adjacent fields in the object. It follows that TSRLM images are to some degree similar and sometimes stronger that in phase microscopy, it depends only on refractive index differences, not on the product of index and thickness, and is fully expressed only when the change in index is abrupt. In very thin details (thickness less than half wavelength) things get more difficult because of interference in thin layers. If important, the study of such thin layers (such as membranes and cell walls) can be improved by the serial use of monochromatic radiations of several different wavelengths.

The TSRLM is not compatible with many of the currently popular ancilliary modes of light microscopy, including the differential contrast enhancement methods. Fluorescence microscopy is fully compatible with TSRLM, however, presenting a method enabling the use of the TSRLM for microchemistry of tissue components of appropriate fluorescence dyes are used [7].

Some remarks on the optical components used in the TSRLM

The demands on the quality of components for tandem scanning are great but we are able to fulfill them. The precision in mechanical manufacture and adjustment of the Nipkow disc and its driving mechanisms and the quality of plane mirrors and the beam splitter do not represent serious problems and can now be achieved consistently after a long development. What can hardly be solved in our laboratories is the development. What can hardly be solved in our laboratories is the development of objectives and eyepieces tailored for use in the TSM.

CONSTRUCTION NOTES

Light sources and illumination optics (one of two sources is used, either)

A tungsten ribbon lamp to provide incoherent of 'critical illumination'. The ribbon is 2 mm broad. A 10x magnified (1st lens) image of the ribbon is projected onto the Nipkow disc. A so called field lens just in front of and nearly in contact with the disc forms the image of the aperture of the 1st lens in the entrance pupil of the microscope objective.

A 'highest pressure' 200 w mercury arc lamp is used to provide coherent illumination. In this case, the first lens (quartz glass) forms an image of the arc into the aperture of the second lens, which in turn forms an image of the first lens aperture in the Nipkow disc (and the adjacent field lens). The combination of the second lens and field lens forms an image of the arc in the entrance pupil of the microscope objective.

A moveable mirror allows switching between the two sources. The sources and their optics are firmly connected with the microscope proper and move with it during coarse focussing.

Nipkow disc

The Nipkow disc is made of 10 μm thick copper foil reinforced on its periphery with a sintered alumina ring 100 mm in diameter. The hole pattern (Fig. 1) is 18 mm broad. The holes are ca.50 μm diameter in TSM2 in Pilsen and in TSM3 in London: they are ca. 30 μm diameter in TSM4 in Zurich. The diameter of the holes changes appropriately from the periphery to the centre of apertured band of the disc. The centration of the disc on the axis of the microscope is achieved by four screws and springs. The axis passes through integral miniature ball bearings.

The electrical driving engine is on the same axis. A specially developed brush and collectorless d.c. motor starts with 200 mw and runs with 50 mw power.

Mirror system

The mirror system is a Nachet configuration which converts central symmetry into virtual identity (Figs 2 and 3). Looking through the opening provided for the attachment of the microscope objective we see both the diametrically opposite fields of the Nipkow disc overlapping and the corresponding holes coincide exactly.

The beam splitter is a 5 μm mica leaf stretched on a 20 mm diameter ring; it has dielectric reflecting coatings.

The tolerances for all distances and angles are so small, that every degree of freedom of any component must be adjusted separately (using very fine pitch screws) following a sophisticated progress schedule. The design is made symmetrical to neutralise thermal expansions and strains.

Optics

Objective lenses

A good correction for chromatic aberration of the objective is required if the microscope is used for fluorescence of for the observation of flat interfaces. All lenses used should have good anti-reflection coatings (in which matter we have found important differences in 'identical' lenses from the same batch from the same reputed manufacturers). Any RMS threaded objective lens can be used.

Immersion lenses are used in preference to suppress surface reflections and to permit the study of rough surfaced objects given suitable matching of refractive indices. It is important to have a good range of magnifications of immersion lenses available, particularly for water, but also for other refractive index media. These are rather difficult to obtain because they are not sold in large numbers.

Dry low power objectives can be used with a cover slip and an immersion medium between it and the object. For thin transparent objects it may be best to use light absorbing supports, again with an immersion medium, in order to minimise reflections from surfaces near the focussed-on plane.

Eyepiece

The widefield eyepieces used must have their focal point beyond the lenses, being focussed on

the Nipkow disc. They should be well corrected for chromatic abberration, field curvature and astigmatism.

Photography and video-recording

Any camera can be used, with its lens focussed to infinity, placed over the eyepiece, and as close to it as convenient. Image intensifiers can be used if necessary, their objectives focussed to infinity.

Focussing

Fine focussing is achieved with a differential screw moving only the objective. Coarse focussing is done by moving the whole microscope up and down, either with an electric motor driven control mechanism, or with a manual coarse focus control. Alternatively, both fine and coarse focus functions can be achieved using a platform (such as the base of a standard optical microscope) on the plinth of the TSRLM.

Stand

The stand of the two most recent versions of the TSRLM has been designed with the prime requirement of providing ample room, and exceptionally tall specimens can be accomodated. A rigid vertical column attached to a massive, ground-flat plinth supports the horizontal arm carrying the microscope and the illumination sources. The height of the microscope proper is adjusted rapidly with the motor drive.

Image quality

Scanning lines are seen in all recorded images with the present microscopes, though they are usually not noticed in routine use of the microscope with the unaided eye. The same pattern is seen regardless of the objective, but this does not degrade the image quality. The scanning lines can be effectively eliminated by using more sophisticated Nipkow disc patterns [15].

Vertical (Z) resolution is improved over conventional microscopes and contrast is higher than in conventional transmitted light,

resembling phase contrast.

Surface colour mode

The chromatic aberration of the objective can be employed to detect differences in Z, and encode measurements of Z, in the surface of opaque objects. Micrographs of surfaces with discrete height steps - such as silicon computer chips - are colour coded contour maps.

TSRLM images are unfamiliar

Interpretation has to be learnt, but experts in a particular field can usually understand images of their own subject matter. This problem is only likely to arise in the case of persons who have only been trained in the histology of stained thin sections.

Depth of penetration beneath surfaces

Limitations on the depth to which one can penetrate into an object an still extract a useful image are provided by several factors:

a) mechanical - the conventional LM immersion objectives are designed to work with a 170 µm coverslip and will collide with the specimen when focussed about 200 µm deep to the surface;

b) optical homogeneity of object;

c) matching of refractive indices of specimen and immersion liquid;

d) the objective aperture;

e) the wavelength of the imaging light.

Greater imaging depths are obtained with red light and infra-red can also be used if a suitable TV camera is available.

Applications

The TSRLM has been applied so far in neurophysiology, neuroanatomy and muscle studies [10,14,18-21] and in morphology of hard tissues, i.e., bone and tooth histology and pathology, bone histomorphometry, studies of dental restorative materials, enamel evolution in many recent and extinct mammalian orders, other vertebrate fossilised mineralised tissues [2,4,5,7,13,15]. We are currently investigating a variety of other potential applications.

THE POSSIBILITY OF A TANDEM SCANNING TRANSMITTED LIGHT MICROSCOPE

It would be interesting to realize also the other possibility, tandem scanning in transmitted light [12b]. The situation here is much more complicated compared with the autocollimation case of reflected light microscopy, but the possibilities of achieving a greatly improved resolution and of employing different contrast enhancing techniques are tempting. The concept may be outlined as follows: the holes of one Nipkow histological section. The light passing through the illuminated points in the object enters the second objective, forming images of these points in the plane of a second Nipkow disc moving synchronously with the first, so that only the light belonging to the geometrical images of the illuminated points is allowed to take part in image formation. In this concept a great freedom exists for independent modifications of any part of the device. The apertures of the objectives can be modified to compress considerably the central parts of diffraction patterns thus leading to better resolution. Phase contrast, interference contrast, polarization, fluorescence, monochromatic absorption, not only in the visible range but also in ultraviolet and infrared, could be employed.

However, the difficulties will be considerable. Both objectives should be extremely well corrected for field curvature and astigmatism, for chromatic aberrations of any kind, and should have equal distortions, to mention only the most important demands. The optical properties of all media between one objective and the observed object plane must be the same as on the side of the other objective. Briefly, to build the tandem scanning transmitted light microscope could be much more difficult than the reflected light case. We are only in the preliminary stages of attacking this problem in terms of theoretical considerations, and would dare to attempt the construction only after all the problems of the TSreflectedLM have been solved.

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